Journal of Chromatography, 356 (1986) 401–408
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 413

ANTIOXIDANT ANALYSIS IN EDIBLE OILS AND FATS BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H. INDYK*
New Zealand Co-operative Dairy Company Ltd., Hamilton (New Zealand)
and
D. C. WOOLLARD
Auckland Dairy Laboratory, Lynfield Agricultural Centre, Ministry of Agriculture and Fisheries, Auckland (New Zealand)
(Received December 12th, 1985)

SUMMARY

Following a simple dilution in the appropriate phase, the sample is injected directly onto either of two normal-phase high-performance liquid chromatography systems (3,5-di-*tert.*-butyl-4-hydroxytoluene or 3-*tert.*-butyl-4-hydroxyanisole–*tert.*-butylhydroquinone) with UV detection at 280 nm. An isocratic ternary mobile phase, incorporating acetonitrile as the polar modifier, has been found to facilitate such an approach, thereby avoiding the discriminatory and recovery problems inherent in other techniques requiring prior sample manipulations. The three most commonly used antioxidants may be estimated at levels down to 3 ppm (3,5-di-*tert.*-butyl-4-hydroxytoluene or 3-*tert.*-butyl-4-hydroxyanisole) and 10 ppm (*tert.*-butylhydroquinone) within 30 min.

INTRODUCTION

Antioxidant properties are expressed by several synthetic phenolic compounds. These include 3,5-di-*tert.*-butyl-4-hydroxytoluene (BHT), 3-*tert.*-butyl-4-hydroxyanisole (BHA), alkyl (propyl, octyl, dodecyl) gallates (PG, OG, DG), *tert.*-butylhydroquinone (TBHQ), nordihydroguairetic acid (NDGA) and 2,4,5-trihydroxybutyrophenone (THBP). They are generally permitted additives in edible oils and fats at levels up to 200 ppm, although precise limits vary in different countries. The tocopherols (α, β, γ, δ) comprise a group of naturally occurring antioxidants and are ubiquitous in all edible oils and fats. Both natural and synthetic phenolics function to delay or prevent oxidative deterioration by interrupting the free radical pathway of lipid autoxidation. Several excellent reviews exist describing this broad topic[1–3].

Many methods exist for the estimation of one or more of the above mentioned inhibitors. Traditionally, colorimetry, spectrophotometry, polarography, paper, thin-layer and more recently gas–liquid chromatography (GLC) have been employed as detection systems subsequent to the extractive techniques of steam distillation or

0021-9673/86/$03.50

solvent partition[4–18]. Many of the GLC techniques require strategies including clean-up techniques[11,12,15] and/or derivatisation prior to injection[11,13]. High-performance liquid chromatography (HPLC) had found favour in this field of analysis and may provide the most facile procedure available. The advantages, compared to other approaches, include specificity, sensitivity, accuracy, precision, multicomponent and automation capabilities and the potential elimination of tedious sample manipulations[19–23]. However, all these reports, in common with a collaborative study[24], necessitate a solvent extraction step followed by optional concentration and reversed-phase chromatography under various isocratic or gradient conditions. These requirements can cause recovery problems and limit the advantages of HPLC. Detection is usually at 280 nm, although in some instances, fluorescence and electrochemical techniques may offer benefits over UV[21,23].

Potential disadvantages to current HPLC methods therefore, remain the manipulatory requirements involved in solvent extraction and the consequent oxidative losses, with the possibility of recovery differences in a multicomponent analysis. In addition, co-extracted residual lipid must be removed before injection onto reversed phase columns. In an attempt to avoid such incidences, this paper reports a procedure for the direct estimation of the most commonly used antioxidants BHT, BHA and TBHQ in oils and fats by normal-phase HPLC. The tocopherols may also be individually estimated under similar conditions.

EXPERIMENTAL

Instrumentation and reagents

The liquid chromatograph used consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 pump, Model U6K injector, Model 440 dual wavelength detector and an Omniscribe dual channel recorder. Later studies included a second pump, Model 680 controller and Hitachi F1000 fluorescence detector.

A Waters 10 μm μPorasil column (30 cm × 3.9 mm I.D.) or Rad-Pak Cyano cartridge fitted in a Waters Z-Module were used for BHA and TBHQ analyses. Two Rad-Pak silica cartridges (5 μm, 8 mm I.D.) in Z-Modules were used in series for BHT work. A Waters Guard-Pak was used with an appropriate protective column insert in each case.

Redistilled petroleum spirit (b.p. 60–80°C), methylene chloride, acetonitrile and 2-propanol (HPLC grade) were employed during the studies. Mobile phases were filtered and degassed prior to use.

All antioxidants were from Sigma (St. Louis, MO, U.S.A.) with the exception of TBHQ (Eastman, Kingsport, U.S.A.).

Procedure

Two eluents were used for routine determinations: for BHA and TBHQ, hexane–methylene chloride–acetonitrile (85.0:9.5:5.5 v/v/v) with either silica or cyanopropyl column; for BHT, hexane–methylene chloride–acetonitrile (88.1:9.8:2.1 v/v/V) with tandem silica system.

Stock solutions of the three antioxidants were freshly prepared in the appropriate mobile phase at various concentrations up to 100 ppm. A ten-fold dilution was used as the working external standard.

Approximately 1 g of the melted oil or fat was accurately weighed into a 10-ml volumetric flask and made to volume with the appropriate mobile phase depending on the HPLC system under use. Gentle warming was sometimes required to assist dissolution of fats keeping the temperature below 40°C. Volumes of 20 μl were injected without further clean up and peak heights compared to those of the standards. For low levels of antioxidants, higher injection volumes were used.

RESULTS

Two types of mobile phase were investigated during attempts to resolve BHA, BHT, TBHQ and the tocopherols from the neutral lipids in oils and fats by normal-phase HPLC. Thus, initial attempts employed a binary hexane–2-propanol system, prompted by its previously reported successes in tocopherol analyses[25–27]. Later, a ternary phase consisting of hexane–methylene chloride–acetonitrile was investigated. In both cases, the proportion of polar modifier was adjusted to optimise resolution. Fig. 1 illustrates the chromatograms obtained for the natural and synthetic standards utilising the two described eluents.

Excellent resolution of standards was obtained with either phase system, the order of elution being opposite to that obtained in reversed-phase chromatography. However, varying the proportion of 2-propanol in the binary eluent had little discriminatory influence on retention behaviour of the natural and synthetic antioxidant classes. Adjustment of acetonitrile in the ternary phase tended to elute preferentially the tocopherol group, while having markedly less impact on the synthetic phenols. Fig. 2 illustrates these observations for the latter phase system.

This capability was further exploited after noting that the binary eluent was incapable of resolving BHA from the congested tocopherol–tocotrienol cluster region in most oil samples. Neither was the hexane–2-propanol phase successful in isolating the early eluting BHT from the neutral lipids, all of which tended to elute at the void volume. Thus, in order to successfully achieve complete resolution of the three synthetic antioxidants from background chromatographic activity, the ternary system was optimised for both BHA–TBHQ and BHT estimations as described.

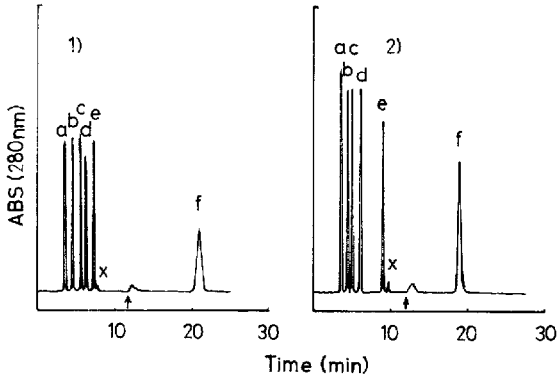


Fig. 1. HPLC separation of antioxidant standards. Conditions: column, μPorasil; mobile phase, (1) hexane–2-propanol (97.5:2.5), (2) hexane–methylene chloride–acetonitrile (85.0:9.5:5.5), flow-rate, 0.8 ml/min increased to 3.0 ml/min at arrow position; detection, 0.1 a.u.f.s. at 280 nm; 20 μl injection. Peaks: a = BHT; b = α-tocopherol; c = γ-tocopherol; d = δ-tocopherol; e = 3-BHA; f = TBHQ; x = 2-BHA (component of 3-BHA additive).

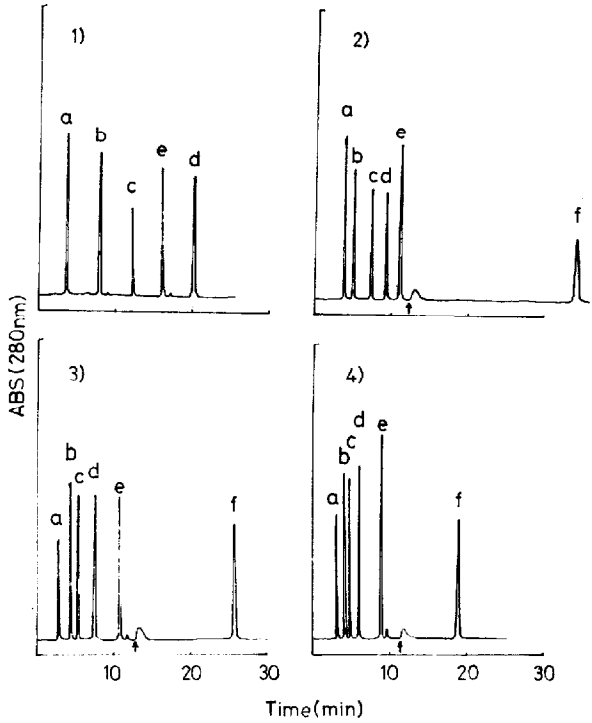


Fig. 2. Influence of acetonitrile on relative retention of antioxidant standards. Conditions and peak identities as in Fig. 1. Mobile phase, hexane–methylene chloride–acetonitrile: (1) 88.7:9.8:1.5; (2) 87.3:9.7:3.0; (3) 85.9:9.6:4.5; (4) 85.0:9.5:5.5.

BHA and TBHQ

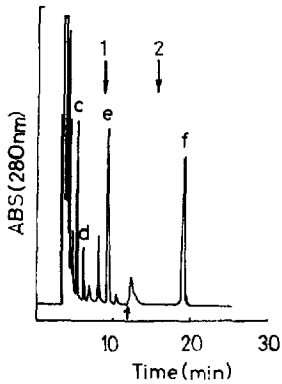
A typical chromatogram of an oil sample containing both antioxidants is reproduced in Fig. 3. A blank oil sample showed no peaks in the elution positions of interest. Confirmation of peak identity was by comparison of spectral ratios using dual wavelength detection at 280 nm and 254 nm and co-elution with authentic antioxidants. Linear calibration curves were obtained for both antioxidants over the required range and coefficients of variation calculated as 2.2% (BHA, $n = 6$) and 4.0% (TBHQ, $n = 6$). Limits of detection (signal-to-noise ratio = 3) for a 20-μl sample injection were 6 ng (BHA) and 20 ng (TBHQ).

Similar results were obtained with the cyanopropyl column under identical chromatography conditions with elution times of approximately half those on silica. Either column was therefore found to be suitable for routine analysis. Table I illustrates these data.

BHT

Optimum conditions for the analysis of the highly lipophilic BHT were established with the tandem silica system and 2.1% acetonitrile ternary phase as shown in Fig. 4.

For routine estimations, it was found expedient for certain samples to investigate the chromatography in the absence of this antioxidant to be certain of no



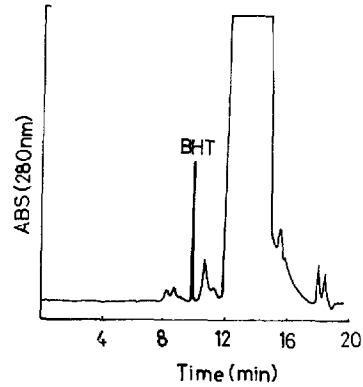


Fig. 3. BHA (75 ppm) and TBHQ (75 ppm) in maize oil. Conditions and identities as in Fig. 1. Diluted sample (20.0 μl) injected at 0.1 a.u.f.s.; attenuated to 0.02 a.u.f.s. (arrow, 1) and 0.005 a.u.f.s. (arrow 2).

Fig. 4. BHT analysis in beef tallow. Conditions: column, tandem Rad-Pak silica (5 μm) in series; mobile phase, hexane–methylene chloride–acetonitrile (88.1:9.8:2.1); flow-rate, 0.8 ml/min; detection, 0.01 a.u.f.s. at 280 nm; 20 μl diluted sample.

spectral interference from non-polar hydrocarbons (carotenes and/or steryl esters).

Several edible oil and fat samples were submitted for analysis by the proposed method and results compared against alternative procedures (Table II).

NDGA, THBP and PG

Several attempts to elute these polyhydroxy phenolics from either silica or cyanopropyl silica columns were unsuccessful with binary or ternary phases containing elevated proportions of the polar modifiers. Peaks were broad, poorly defined and hence were unsuitable for quantitation. Programmed solvent gradients also failed to achieve a satisfactory result.

DISCUSSION

There have been several reports of topopherol congener analysis by direct injection of the diluted lipids on a silica column HPLC system[25–27]. In view of functional group similarities between tocopherols and the synthetic antioxidants, it was decided to investigate the same approach to the latter group of food additives. Al-

TABLE I

ANTIOXIDANTS (ppm) BY CYANOPROPYL AND SILICA NORMAL-PHASE CHROMATOGRAPHY

Mobile phase: hexane–methylene chloride–acetonitrile (85.0:9.5:5.5). Data are mean of duplicates.

Oil or fat	*Antioxidant*	*CN*	*SiOH*
Palm	TBHQ	42.4	44.4
Soya	TBHQ	38.2	40.4
Beef tallow	TBHQ	35.1	32.3
Beef tallow	BHA	133.3	134.5
Beef tallow	BHA	66.7	64.0

TABLE II

COMPARISON OF METHODS FOR ANTIOXIDANTS (ppm)

Oil or fat	*Antioxidant*	*Proposed method**	*Alternative HPLC***	*GLC****	*Spectrometric*§*	*Label claim*
Palm	TBHQ	43.4	42.6	–	47.0	48.0
Soya	TBHQ	39.3	–	–	50.0	48.0
Tallow	TBHQ	33.7	34.2	–	43.0	48.0
Tallow	BHA	134.0	149.0	148.0	–	100.0
Tallow	BHA	65.4	–	–	–	100.0
Maize	BHA	70.8	72.0	–	–	100.0
Tallow	BHT	48.0	44.2	38.0	–	50.0

* Silica column.

** Methanol extraction; gradient reversed phase (ref. 20).

*** Sample dissolved in hexane; 1 μl on OV-101 at 150°C (ref. British Standard method No. 684 - Sect. 2.36, 1983 (1s0-6463, 1982) determination of BHA, BHT; *GLC* method).

§ Eastman-Kodak Standard Procedure No. 49.

though binary phases of hexane–2-propanol were found to be suitable when TBHQ only was required for estimation, such a system was not applicable to either BHA or BHT, where serious chromatographic interferences were observed, with BHA eluting close to the tocopherols and the poorly retained BHT masked by the solvent front. In other reports, where the emphasis was on tocol or neutral lipid estimation, similar interference problems from BHA[27] and BHT[28] have been noted. To the current authors knowledge, there has been only one report of a normal-phase technique specifically designed for antioxidant analysis, where a binary phase of hexane-dioxane was successful for TBHQ, while BHT and BHA failed to be resolved from other artefacts present in oils or fats[29].

Acetonitrile has previously been reported to assist in the chromatographic separation of cholesteryl esters, triglycerides and fatty acids as part of an isocratic quaternary phase consisting also of hexane–*n*-butyl chloride–acetic acid[30]. In the present work, where low wavelength UV detection was not required, a miscible and successful phase system was established by substituting methylene chloride for *n*-butyl chloride. Compared to binary solvent systems without acetonitrile, resolution between neutral triglycerides and non-polar hydrocarbons was greatly improved using the described mobile phase, with partial resolution of triglycerides also achieved. It has been commented[30] that acetonitrile, as the polar component in a normal-phase system, may allow some unique separations involving both unsaturated centres and carbon chain length. Of greater current interest however, was the advantage gained in antioxidant analyses, particularly in facilitating resolution of BHA and BHT from background. It seems likely that the polar, but non-hydrogen bonding nature of the proposed ternary system, significantly influences the elution character of the relatively lipophilic vitamin E congeners as compared to the mono- and dihydroxy phenolics (BHT, BHA, TBHQ) all of which lack the lipophilic sidechain of the tocopherols. The major consequence is therefore the ability to manipulate the phase to favour elution in regions devoid of chromatographic activity. Under certain conditions, it may also be appropriate to optimise this phase system for dedicated tocopherol estimations, where benefits over the more traditional binary eluents may be gained.

A small peak associated with authentic BHA and BHA-supplemented samples may well be 2-BHA. This has been reported previously as the minor structural isomer with 3-BHA in most preparations[31]. The two isomers have been previously resolved on an aminopropyl column with a binary hexane–2-propanol phase, although a successful separation on underivatised silica was not reported[32]. Perhaps this is a further illustration of the potential advantages of the presently reported acetonitrile modified system.

In an attempt to improve sensitivity and selectivity, fluorescence detection of the phenolics was investigated. Preliminary experiments established that both BHA and TBHQ fluoresce with reasonable quantum yields but that BHT was an exceptionally poor fluorophore (presumably a consequence of steric deformation). Such observations support other published information[7,8,21,23,29] and indicate that significant benefits are not gained as compared to UV detection. Electrochemical detection provides a potentially more sensitive system[21,23] but requires a conductive electrolyte common to reversed-phase chromatography and would not be applicable to the proposed non-aqueous system.

While excellent reproducibility has been observed consistently with a silica column, cyanopropyl-derivatised silica was also investigated as an alternative. Silica has well recognised limitations attributed mainly to its reportedly variable degree of hydration. This property is capable of disturbing elution volumes and may be minimised when the silanyl hydroxy groups are protected by derivatisation. When used in a normal-phase mode, the cyano column can yield similar results without the problem of moisture contamination. Elution volumes, under similar conditions are however, significantly less than on silica, reflecting the reduced interaction of the antioxidant solutes on the less active cyano surface. The cyano column has previously been found useful in neutral lipid chromatography employing binary eluents[28].

Currently, the chromatographic protocol favoured in this laboratory is the use of a simple isocratic flow programme as an alternative to the step program described earlier for BHA and TBHQ. This approach avoids baseline disturbance associated with the sudden pressure surge consequent to a manual step change. A 0.5–4.0 ml/min linear flow program over 20 min has been found satisfactory.

There are several reports of reversed-phase LC procedures for antioxidant or multiantioxidant determinations in edible oils and fats. These methods require that the phenolics are isolated from the lipid matrix in an extraction step. Often, discrimination of recovery between analytes is unavoidable due to the extreme range of polarities and separate extraction strategies have been recommended in addressing this problem[19,22]. Others have commented specifically on the poorer recovery of BHT[14,20,23,24]. Of particular concern is the recognition that certain antioxidants, most notably TBHQ, oxidise readily once isolated and serious recovery losses can and do occur[14,17,24]. It seems apparent that the technique proposed presently avoids such problems, since no manipulation of the sample other that dilution is required.

The method does, however, fail when extended to other antioxidants. Thus, NDGA, THBP and PG could not be eluted with acceptable peak shape with either column type or phase, while solubility was an additional problem. Consideration of the tri- and tetrahydroxy-substituted functionality and consequent high polarity of these phenolics, reveals that this limitation is probably unavoidable. Mediating against this constraint, however, is the recognition that BHT, BHA and TBHQ rep-

resent greater than 90% of synthetic antioxidants in commercial use in the edible oils industry. Thus, the gallate esters, NDGA and THBP all suffer from low solubility in oils and often are the cause of serious discoloration[1,33]. The latter two are further rarely available due to cost. Indeed, while BHT and BHA are traditionally favoured, TBHQ seems to be the more useful and potent synthetic antioxidant, exhibiting a protective influence greater than any of the other primary antioxidants and second only to natural γ-tocopherol at the 200 ppm level[33].

ACKNOWLEDGEMENTS

The authors thank the New Zealand Co-operative Dairy Company Ltd, and NZ Ministry of Agriculture and Fisheries, Dairy Division, for facilities provided. Appreciation is extended to D. Fenton and L. Eyres of Abels Ltd. for their enthusiastic support of this project.

REFERENCES

1 E. R. Sherwin, *J. Am. Oil Chem. Soc.*, 53 (1976) 430.
2 T. Richardson and M. Koryeka-Dahl, in P. F. Fox (Editor), *Developments in Dairy Chemistry*, Vol. 2, Applied Science Publ., Barking, 1983, Ch. 7, p. 241.
3 T. P. Labuza, *Crit. Rev. Food Tech.*, 2 (1971) 355.
4 W. Horowitz (Editor), *Official Methods of Analysis of the Association of Official Analytical Chemists*, Association of Official Analytical Chemists, Washington, DC, 1975, Ch. 20, p. 348.
5 *IUPAC Standard Methods for the Analysis of Oils, Fats and Derivatives*, 6th ed., Part 1; 2.621, 2.622, Pergamon, Oxford, 1979, p. 164.
6 S. Jayaraman, T. S. Vasundhara and D. B. Pariaar, *Mikrochim. Acta*, 11 (1976) 365.
7 H. W. Latz and R. J. Hurtubise, *J. Agric. Food Chem.*, 17 (1969) 352.
8 S. Dilli and K. Robards, *Analyst*, 102 (1977) 201.
9 M. Nakazato, M. Kanmuri, T. Takanari, K. Fujinuma and Y. Naoi, *Shokuhin Eiseigaku Zasshi*, 22 (1981) 239.
12 K. D. Wadsworth and R. Basscttc, *J. Food Protection*, 48 (1985) 487.
11 E. E. Stoddard, *J. Assoc. Off. Anal. Chem.*, 55, 5 (1972) 1081.
12 J. Singh and M. R. Lapointe, *J. Assoc. Off. Anal. Chem.*, 57, 4 (1974) 804.
13 R. E. Austin and D. M. Wyatt, *J. Am. Oil Chem. Soc.*, 57 (1980) 422.
14 D. M. Wyatt, *J. Am. Oil Chem. Soc.*, 58 (1981) 917.
15 D. B. Min, D. Ticknor and D. Schweizer, *J. Am. Oil Chem. Soc.*, 59 (1982) 378.
16 D. B. Min and D. Schweizer, *J. Food Sci.*, 48 (1983) 73.
17 T. Stijve, *Dtsch. Lebensm.-Rundsch.*, 79 (1983) 108.
18 L. Z. Yu, M. Inoko and T. Matsuno, *J. Agric. Food Chem.*, 32 (1984) 681.
19 K. J. Hammond, *J. Assoc. Public Analysts*, 16 (1978) 17.
20 A. W. Archer, *Anal. Chim. Acta*, 128 (1981) 235.
21 A. N. Masoud and Y. N. Cha, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 5 (1982) 299.
22 D. C. Woollard, *N.Z.J. Dairy Sci. Tech.*, 18 (1983) 55.
23 Y. Kitada, Y. Ueda, M. Yamamoto, K. Shinomiya and H. Nakazawa, *J. Liquid Chromatogr.*, 8 (1985) 47.
24 B. D. Page, *J. Assoc. Off. Anal. Chem.*, 66 (1983) 727.
25 P. Taylor and P. Barnes, *Chem. Ind.*, 20 (1981) 722.
26 F. Zonta and B. Stancher, *La Rivista Italiana Delle Sostanze Grasse*, 60 (1983) 195.
27 J. N. Thompson and G. Hatina, *J. Liquid Chromatogr.*, 2 (1979) 327.
28 D. N. Palmer, M. A. Anderson and R. D. Jolly, *Anal. Biochem.*, 140 (1984) 315.
29 P. J. Van Niekerk and L. M. Du Plessis, *J. Chromatogr.*, 187 (1980) 436.
30 J. G. Hamilton and K. Comai, *J. Lipid Res.*, 25 (1984) 1142.
31 L. W. Wattenberg, J. B. Coccia and L. K. T. Lam, *Cancer Res.*, 40 (1980) 2820.
32 G. A. S. Ansari, *J. Chromatogr.*, 262 (1983) 393.
33 K. G. Berger, *J. Am. Oil Chem. Soc.*, 62 (1985) 434.